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Protective effects of xanthohumol against the genotoxicity of heterocyclic aromatic amines MeIQx and PhIP in bacteria and in human hepatoma (HepG2) cells

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ABSTRACT

Previous studies showed that xanthohumol (XN), a hop derived prenylflavonoid, very efficiently protects against genotoxicity and potential carcinogenicity of the food borne carcinogenic heterocyclic aromatic amine (HAA) 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). In this study, we showed that XN was not mutagenic in *Salmonella typhimurium* TA98 and did not induce genomic instability in human hepatoma HepG2 cells. In the bacteria XN suppressed the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8 dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) induced mutations in a dose dependent manner and in HepG2 cells it completely prevented PhIP and MeIQx induced DNA strand breaks at nanomolar concentrations. With the QRT-PCR gene expression analysis of the main enzymes involved in the biotransformation of HAAs in HepG2 cells we found that XN upregulates the expression of phase I (*CYP1A1* and *CYP1A2*) and phase II (*UGT1A1*) enzymes. Further gene expression analysis in cells exposed to MeIQx and PhIP in combination with XN revealed that XN mediated up-regulation of *UGT1A1* expression may be important mechanism of XN mediated protection against HAAs induced genotoxicity. Our findings confirm the evidence that XN displays strong chemopreventive effects against genotoxicity of HAAs, and provides additional mechanistic information to assess its potential chemopreventive efficiency in humans.

1. Introduction

Epidemiological studies suggest that diet plays an important role in ethiology of human cancer (Doll and Peto, 1981). On one hand human diet often contains ingredients that cause DNA damage and are potentially carcinogenic, and on the other hand it contains numerous natural constituents with protective effects against cancer and other mutation-related diseases (Ferguson et al., 2004; Knasmuller et al., 2002; Zegura et al., 2011). An important class of compounds in the diet that are considered a dietary risk factor for human cancer are heterocyclic aromatic amines (HAAs) that are regularly formed in cooked meat products (Skog et al., 1998). Since their discovery 30 years ago, more than 20 HAAs have been identified in cooked meat, fish and poultry (Turesky, 2010), with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8 dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) being the most abundant (Costa et al., 2009; Polak et al., 2009; Salmon et al., 2006). HAAs are genotoxic carcinogens, and animal studies showed that they induce cancer in multiple species at multiple tissues (Sugimura et al., 2004). Several human epidemiological studies also indicated association between intake of HAAs and risk for important types of human cancer such as breast, colon or pancreas in meat-eating populations (Zheng and Lee, 2009). The International Agency for Research on Cancer (IARC) classified several HAAs as *probable* or *possible* human carcinogens (IARC, 1993).

Natural phytochemicals derived from dietary sources or medicinal plants have gained significant recognition in the potential management of several human clinical conditions including cancer. Xanthohumol (XN, 3′-[3,3-dimethyl allyl]-2,4,4′-trihydroxy-6′-methoxychalcone), the principal prenylated flavonoid present in the hop plant, *Humulus lupulus* L. (Yilmazer et al., 2001a; Stevens and Page, 2004), has been characterized as a potential "broad-spectrum" cancer chemo-preventive agent acting by multiple mechanisms in the initiation, promotion and progression stage of cancer development (Gerhauser et al., 2002). The antimutagenic effect of XN has been first shown in a bacterial test system with *Salmonella typhimurium* against the HAA 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (Miranda et al., 2000a). In the test systems with metabolically active human hepatoma HepG2 cells and precision-cut rat liver slices, XN completely prevented formation of IQ and benzo(a)pyrene (BaP) induced DNA damage at concentrations as

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low as 10 nM (Plazar et al., 2007, 2008). XN showed protective effects also against oxidative DNA damage induced by *tert*-butyl hydroperoxide (*t*-BOOH) (Plazar et al., 2007, 2008), and menadione (Dietz et al., 2005). In addition, XN exhibited anti-inflammatory and antiproliferative effects at the promotional stage of carcinogenesis and prevented formation of carcinogen induced preneoplastic lesions in liver colon and mammary gland (Ferk et al., 2010; Gerhauser et al., 2002).

The protective effects of XN against IQ induced genotoxicity has been extensively studied, however there is no data on potential protective effect of XN against genotoxicity of other HAAs. Therefore, in this study we evaluated the potential protective effect of XN against the most abundant HAAs formed in fried and grilled meat, fish and poultry: PhIP and MeIQx. The protective potential of XN against PhIP and MeIQx induced genotoxicity was assessed in the bacterial test system with *S. typhimurium* TA98 and in the test system with human hepatoma HepG2 cells with the comet and cytokinesis block micronucleus (CBMN) cytome assay. To shed light whether the mechanism that account for the antigenotoxic effect of XN involves modulation of metabolism of HAAs, we evaluated the influence of XN on gene expression of the main enzymes involved in the biotransformation of MeIQx and PhIP in HepG2 cells. The test system with HepG2 cells has been shown to be particularly convenient for investigations of dietary anti-mutagens. Because these cells retain the activities of many metabolic enzymes in inducible form they enable detection of protective mechanisms that are not present in most of the conventional *in vitro* models (Knasmuller et al., 1998, 2002; Mersch-Sundermann et al., 2004).

2. Materials and methods

2.1. Chemicals

Williams' medium E, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT [CAS 298-93-1]), cytochalasin B (Cyt-B), acridine orange (AO), dimethyl sulfoxide (DMSO [CAS 67-68-5]), EDTA [CAS 6381-92-6], ethidium-bromide solution (EtBr [CAS 1239-45-8]), Triton X-100 and benzo(a)pyrene [CAS 50-32-8], were obtained from Sigma–Aldrich (St. Louis, USA). Penicillin/streptomycin, fetal bovine serum (FBS), L-glutamine, phosphate-buffered saline (PBS) were from Euro Clone (Siziano, Italy) and trypsin from BD (Franklin Lakes, USA). PhIP and MeIQx were from Toronto Research Chemicals (Ontario, Canada); Xanthohumol was from the Nookandeh-Institut für Naturstoffchemie GmbH (Homburg/Saar, Germany). Normal melting-point (NMP) and low melting point (LMP) agarose [CAS 9012-36-6] and TRIzol® reagent were from Gibco BRL (Paisley, Scotland); High Capacity cDNA Archive Kit and Taqman Gene Expression Assays were from Applied Biosystems, Forest City, CA, USA, TaqMan Universal PCR Master Mix from Applied Biosystems, Branchburg, NJ, USA, and Human GAPDH from Applied Biosystems, Warrington, UK. Lyophilized Aroclor 1254 induced male rat liver post-mitochondrial fraction (S9) was obtained from Moltox, Boone, USA. All chemical reagents were of the purest grade available and all solutions were made using Milli-Q water.

XN, PhIP, MeIQx and BaP were dissolved in DMSO; the final concentration of DMSO in incubation mixtures was not higher than 1%.

2.2. Mutagenicity and antimutagenicity testing with the Salmonella/microsomal (Ames) assay

The mutagenicity/antimutagenicity studies were performed with the Salmonella/microsomal reverse mutation assay (Maron and Ames, 1983). For the mutagenicity testing 100 μl XN (final concentrations 2.5, 5, 10 and 20 μg/plate), 100 μl overnight culture of *S. typhimurium* strain TA98 and 500 μl of 4% S9 mix were added to 2 ml of molten top agar, mixed and poured onto minimal agar plates. For the antimutagenicity assay, 100 μl XN (final concentrations 2.5, 5, 10 and 20 μg/plate), 100 μl overnight culture of *S. typhimurium* strain TA98 and 500 μl of 10% S9 mix were added to 2 ml of molten top agar containing MeIQx (final concentration 0.4 μM/plate) or PhIP (final concentration 4 μM/plate), mixed and poured onto minimal agar plates. The number of His^+ revertants was scored after incubation for 72 h at 37 °C. Three plates were used per experimental point.

2.3. Human hepatoma HepG2 cells

HepG2 cells were provided by Prof. Firouz Darroudi, Department of Radiation Genetics and Chemical Mutagenesis, University of Leiden, The Netherlands. The cells were grown in Williams' medium E containing 15% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin at 37 °C in 5% CO_2. Cells were used at passages between 6 and 12.

2.4. Cytotoxicity assay

Cytotoxicity of XN was determined with the MTT assay, according to Mossmann (1983) with minor modifications. This assay measures the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble formazan by dehydrogenase enzymes of intact mitochondria of living cells. HepG2 cells were seeded onto a 96-well microplate at a density of 7500 cells/well in 200 μl. The next day, the growth medium was replaced by complete growth medium, containing 0.1, 1, 10, 50 and 100 μM XN and incubated for 24 h. At the end of the exposure, the cells were washed with PBS and the medium was replaced by fresh growth medium. MTT was then added to a final concentration of 0.5 mg/ml. After 3 h, the medium was removed and the formazan crystals were dissolved in DMSO. The amount of formazan crystals directly correlates to the number of viable cells. The optical density (OD) was measured at 570 nm (reference filter 690 nm) using a spectrofluorimeter (Tecan, Genios). Relative cell survival (viability) was calculated by dividing the OD of the treated cells with the OD of the control cells. The cytotoxicity was measured in two independent experiments each time with five replicates per treatment point.

2.5. HepG2 cells treatment for the comet assay, CBMN cytome assay and QRT-PCR gene expression analysis

Genotoxicity, antigenotoxicity and the effects of mRNA expression of selected genes in HepG2 cells were determined using the same treatment conditions. The cells were exposed to XN alone (0, 0.01, 0.1, 1 or 10 μM) or in the combination with either 200 μM PhIP or 250 μM MeIQx for 24 h at 37 °C in 5% CO_2. 1% DMSO was used as the negative solvent control and BaP (30 μM) was as the positive control. At the end of the exposure the genotoxicity and antigenotoxicity were determined by the comet assay and by cytokinesis block micronucleus assay (CBMA), and mRNA expression of metabolic enzymes was determined with the QRT-PCR. The experiments were performed in three independent repetitions.

2.6. Comet assay

At the end of the exposure, the cells were washed with PBS, trypsinized, centrifuged at 115*g* for 5 min and resuspended in fresh medium. The comet assay was performed according to Singh et al. (1988). Briefly, 30 μL of cell suspension was mixed with 70 μL 1% LMP agarose and added to fully frosted slides coated with 80 μL of 1% NMP agarose. Subsequently, the cells were incubated in a lyses solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for at least 1 h at 4 °C, then the slides were placed into an alkaline solution (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min at 4 °C to allow DNA unwinding and electrophoresed for 20 min at 25 V (300 mA). Finally, the slides were neutralized in 400 mM Tris buffer (pH 7.5) for 15 min, stained with EtBr (5 μg/ml) and analyzed using a fluorescence microscope under 400x magnification (Nikon, Eclipse 800). Images of fifty randomly selected nuclei per experimental point were analyzed with the image analysis software (Comet Assay IV, Perceptive Instruments, UK). For each sample, three independent experiments were performed. The results from three independent experiments are expressed as % of tail DNA and are shown as box plots. One-way analysis of variance (ANOVA, Kruskal–Wallis) was used to analyze the differences between the treatments within each experiment. Dunnett's test was used for multiple comparison of treated versus the control cells; $p < 0.05$ was considered as statistically significant (∗).

2.7. Cytokinesis block micronucleus (CBMN) cytome assay

The CBMN cytome assay was performed according to Fenech (2000, 2006) with minor modifications (Straser et al., 2011). At the end of the exposure the treatment medium was removed and the cells were washed twice with PBS. Then the medium containing Cyt-B (final concentration 2 μg/ml) was added and the cells were incubated at 37 °C/5% CO_2 for additional 26 h. The cells were then trypsinised, washed with PBS, incubated in cold hypotonic solution (75 mM KCl) for 5 min and fixed with methanol/acetic acid (3/1) (v/v) and formaldehyde. Subsequently, the cells were put on microscope slides and air dried. All slides were randomised and coded prior to analysis. The slides were stained with acridine orange (20 μg/ml), and examined under the fluorescence microscope (Eclipse 800, Nikon, Japan) at 400× magnification. For each experimental point the total number of micronuclei (MNi), cells containing micronucleus (MNed cells), nucleoplasmic bridges (NPB) and nuclear buds (NB) were counted in 1000 binucleated cells (BNC) according to the criteria described by Fenech (2000). The nuclear division index (NDI) was estimated by scoring 500 cells with one to four nuclei. The NDI was calculated using the formula [M1 + 2M2 + 3(M3 + M4)]/1000, where M1–M4 represent the number of cells with one to four nuclei, respectively. Statistically significant differences between the number of MN, NPB or NB in treated and control groups was determined by Student's *t*-test ; $p < 0.05$ was considered as statistically significant.

2.8. Real-time quantitative PCR (QRT-PCR) analysis

After the incubation, the cells were washed with 1x PBS and total RNA was isolated using TRIzol® reagent, according to the manufacturer's protocol with minor modifications. Glycogen (20 μg/ml) was added to the cell lysate. The RNA was incubated with isopropyl alcohol overnight at −20 °C to precipitate. All solutions needed for RNA isolation were prepared in RNase-free water.